MDR-LIKE ABC TRANSPORTER GENE FROM PLANTS

This application is a continuation in part of International Application No. PCT/US99/22363, filed September 24, 1999, which claims priority under 35 U.S.C. §120 to U.S. Provisional Application 60/101,814, the entireties of both of which are incorporated by reference herein.

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the National Science Foundation, Grant No. IBN-9416016.

FIELD OF THE INVENTION

This invention relates to the field of stress resistance in plants. In particular, the invention provides a novel gene from plants, which encodes an MDR-like ABC transporter, involved in detoxification of certain xenobiotics to protect plants from their detrimental effects.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application to describe the state of the art to which the invention pertains. Each of these publications is incorporated by reference herein.

Environmental stress is one of the most important limitations on plant productivity, growth and survival. An ever-increasing source of environmental stress to plants is

the stress caused by environmental pollutants in the soil, water and atmosphere. Such pollutants include herbicides, pesticides and related agronomic products, as well as organic and inorganic waste material from industry and other sources. Other toxic agents that threaten the survival of plants include various toxins produced by ephiphytic or soilborne microorganisms, such as fungi and bacteria.

In order to survive in toxic environments, plants must have mechanisms to detoxify xenobiotics, heavy metals and other toxic compounds. This generally involves modification of the toxic compound and subsequent excretion into the vacuole or apoplastic space. Recently, certain ATP-binding cassette (ABC) transporters have been identified in plants, which appear to be involved in the detoxification process.

The ABC transporter family is very large, with representatives existing in many different classes of organisms. Two well studied groups of ABC transporters, encoded by *mdr* and *mrp* genes, respectively, are associated with the multi-drug resistance phenomenon observed in mammalian tumor cells. The *mdr* genes encode a family of P-glycoproteins that mediate the energy-dependent efflux of certain lipophilic drugs from cells. The *mrp* genes encode a family of transporters that mediate the extrusion of a variety of organic compounds after their conjugation with glutathione. *YCF1*, the yeast homolog of *mrp*, encodes a protein capable of glutathione-mediated detoxification of heavy metals.

Homologs of *mrp* and *mdr* genes have been identified in plant species. In *Arabidopsis thaliana*, the glutathione-

conjugate transporter encoded by the *mrp* homolog is located in the vacuolar membrane and is responsible for sequestration of xenobiotics in the central vacuole (Tommasini et al., FEBS Lett. 411: 206-210, 1997; Li et al., Plant Physiol. 107: 1257-1268, 1995). An *mdr*-like gene (*atpgp1*) has also been identified in *A. thaliana*, which encodes a putative P-glycoprotein homolog. The *atpgp1* gene was found to share significant sequence homology and structural organization with human *mdr* genes, and was expressed with particular abundance in inflorescence axes (Dudler & Hertig, J. Biol. Chem. 267: 5882-5888, 1992). Other MDR homologs have been found in potato (Wang et al., Plant Mol. Biol. 31: 683, 1996) and barley (Davies et al., Gene 199: 195, 1997).

The aforementioned *mrp* and *mdr* plant homologs were identified as a result of an effort to understand the molecular basis for development in plants of cross-resistance to herbicides of unrelated classes. However, these transporters are likely to serve the more general role in plants of sequestering, secreting, or otherwise detoxifying various organic and inorganic xenobiotics. Accordingly, it will constitute an advance in the art of plant genetic engineering of stress tolerance to identify and characterize other members of this class of transporters in plants.

SUMMARY OF THE INVENTION

In accordance with the present invention, a new plant *mdr* homolog, referred to herein as *plPAC*, has been identified. Unlike the previously identified plant *mdr* homologs, this new gene is inducible by a class of compounds known to inhibit chloride ion channels. The new gene is also

inducible by auxin and binds NPA.

According to one aspect of the invention, a nucleic acid isolated from a plant is provided, which encodes a p-glycoprotein that is inducible by exposure of the plant to NPPB or auxin. The isolated nucleic acid is preferentially expressed in plant roots upon exposure of the plant to NPPB. In a preferred embodiment, the plant from which the nucleic acid is isolated is selected from the group consisting of *Brassica napus* and *Arabidopsis thaliana* and is 3850-4150 nucleotides in length. In a more preferred embodiment, the nucleic acid has the restriction sites shown in Figure 4 for at least three restriction enzymes. In particularly preferred embodiments, the nucleic acid molecule encodes a polypeptide having SEQ ID NO:2. In an exemplary embodiment, the nucleic acid is a cDNA comprising the coding region of SEQ ID NO:1 or SEQ ID NO:10.

According to another aspect of the invention is an expression cassette that comprises a plPAC gene operably linked to a promoter, and in a more preferred embodiment the plPAC gene is from *Arabidopsis*. In preferred embodiments, the expression cassette comprises the cauliflower mosaic virus 35S promoter, and part of all of SEQ ID NO:1 or SEQ ID NO:10. Further included in this aspect is a vector comprising the expression cassette and a method for producing transgenic plants with the expression cassette and vector.

Another aspect of the invention is drawn to transgenic cells and plants containing the nucleic acids of the invention. In one preferred embodiment, the nucleic acids are be in the aforementioned expression cassette. Further included in this aspect are reproductive units from

the transgenic plant.

According to another aspect of the invention, an isolated nucleic acid molecule is provided, which has a sequence selected from the group consisting of: a) SEQ ID NO:1 and SEQ ID NO:10; b) a nucleic acid sequence that is at least about 60% homologous to the coding regions of SEQ ID NO:1 or SEQ ID NO:10; c) a sequence hybridizing with SEQ ID NO:1 or SEQ ID NO:10 at moderate stringency; d) a sequence encoding part or all of a polypeptide having SEQ ID NO:2; e) a sequence encoding an amino acid sequence that is at least about 70% identical to SEQ ID NO:2; f) a sequence encoding an amino acid sequence that is at least about 80% similar to SEQ ID NO:2; g) a sequence encoding an amino acid sequence that is at least about 40% similar to residues 1-76, 613-669 or 1144-1161 of SEQ ID NO:2; and h) a sequence hybridizing at moderate stringency to a sequence encoding residues 1-76, 613-669 or 1144-1161 of SEQ ID NO:2. A polypeptide produced by expression of the above listed sequences is also provided.

According to another aspect of the invention, an isolated plant p-glycoprotein, which is inducible upon exposure of the plant to NPPB, is provided. The polypeptide preferably confers upon a cell in which it is found resistance to Rhodamine 6G. The polypeptide is preferentially produced in roots upon the exposure to the NPPB. The polypeptide is preferably from *Brassica napus* or *Arabidopsis thaliana*. In most preferred embodiments, the polypeptide has a sequence that is a) an amino acid sequence that is at least 80% similar to SEQ ID NO:2; b) an amino acid sequence that is at least 70% identical to SEQ ID NO:2; c) an amino acid sequence that is at least 40% similar to residues

1-76, 613-669 or 1144-1161 of SEQ ID NO:2; and d) an amino acid sequence encoded by a nucleic acid sequence hybridizing at moderate stringency to a amino acid sequence encoding residues 1-76, 613-669 or 1144-1161 of SEQ ID NO:2.

According to other aspects of the invention, antibodies immunologically specific for the polypeptides of the invention are provided, that immunologically specific to any of the polypeptides, of polypeptide encoded by the nucleic acids of the invention. In a preferred embodiment, the antibody is immunospecific to residues 1-76, 613-669 or 1144-1161 of SEQ ID NO:2.

According to another aspect of the invention, a plant p-glycoprotein gene promoter, which is inducible by NPPB, is also provided. In a preferred embodiment, the promoter is part or all of residues 1-3429 of SEQ ID NO:10. According to another aspect of the invention, plants that have reduces levels of plPAC protein are provided. In a preferred embodiment, these plants have mutations in the *plPAC* gene, and in a particularly preferred embodiment, the *plPAC* gene is mutated due to the insertion of a T-DNA. Also provided with this aspect is a method for selecting plants with mutations in *plPAC* using SEQ ID NOS:11-14 as PCR primers.

These and other features and advantages of the present invention will be described in greater detail in the description and examples set forth below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Amino acid sequence lineup of *ATPAC* deduced amino acid sequence and the amino acid sequences of

related mammalian and plant genes. The lineup shows the ATPAC deduced amino acid sequence (SEQ ID NO:2) compared with (1) hmdr1 (SEQ ID NO:3); (2) mmdr1 (SEQ ID NO: 4); (3) hmdr3 (SEQ ID NO:5); (4) mmdr2 (SEQ ID NO:6); (5) atpgp1 (SEQ ID NO:7); and (6) atpgp2 (SEQ ID NO:8). A consensus sequence (SEQ ID NO: 9) is also shown.

Figure 2. Graph depicting the effect of rhodamine 6G on the growth rate of cells transformed with and expressing *ATPAC* as compared with control cells not containing *ATPAC*.

Figure 3. Restriction map of genomic clone of *ATPAC*, SEQ ID NO:10.

Figure 4. Restriction map of cDNA clone of *ATPAC*, SEQ ID NO:1.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specification and claims.

With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also

comprise a cDNA molecule.

With respect to RNA molecules of the invention the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below). Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus define the differences. For purposes of this invention, the DNAStar program (DNAStar, Inc., Madison, Wisconsin) and the default parameters used by that program are the parameters intended to be used herein to compare sequence identity and similarity. Alternately, the Blastn and Blastp 2.0 programs provided by the National Center for Biotechnology Information (at http://www.ncbi.nlm.nih.gov/blast/ ; Altschul et al., 1990, J Mol Biol 215:403410) using a gapped alignment with default parameters, may be used to determine the level of identity and similarity between nucleic acid sequences and amino acid sequences.

The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nature of the protein (i.e. the structure, thermostability characteristics and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved

sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

With respect to protein, the term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by

expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

With respect to antibodies of the invention, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

With respect to oligonucleotides, the term "specifically hybridizing" refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of

the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

The term "expression cassette", as used herein, comprises 5' and 3' regulatory regions operably linked to a coding sequence. The coding sequence may be in the sense or antisense orientation with respect to the 5' regulatory region.

The term "promoter region" refers to the 5' regulatory regions of a gene.

The term "reporter gene" refers to genetic sequences which may be operably linked to a promoter region forming a transgene, such that expression of the reporter gene coding region is regulated by the promoter and expression of the transgene is readily assayed.

The term "selectable marker gene" refers to a gene product that when expressed confers a selectable phenotype, such as antibiotic resistance, on a transformed cell or plant.

The term "operably linked" means that the regulatory sequences necessary for expression of the coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of coding sequences and transcription control elements (e.g. promoters, enhancers, and termination elements) in an expression vector.

The term "DNA construct" refers to genetic sequence used to transform plants and generate progeny transgenic plants. These constructs may be administered to plants in a viral or plasmid vector. Other methods of delivery such as

Agrobacterium T-DNA mediated transformation and transformation using the biolistic process are also contemplated to be within the scope of the present invention. The transforming DNA may be prepared according to standard protocols such as those set forth in "Current Protocols in Molecular Biology", eds. Frederick M. Ausubel et al., John Wiley & Sons, 2001.

The term "xenobiotic" refers to foreign chemicals or agents not produced or naturally found in the organism. The term is commonly used in reference to toxic or otherwise detrimental foreign chemicals, such as organic pollutants or heavy metals.

II. Description of *plPAC* and its Encoded Polyeptide

In accordance with the present invention, a nucleic acid encoding a novel ATP-binding-cassette (ABC) transporter has been isolated and cloned from plants. This novel ABC transporter is induced by auxin and binds NPA. The nucleic acid is referred to herein as *plPAC*.

A cDNA clone of the *plPAC* from *Arabidopsis thaliana*, an exemplary *plPAC* of the invention, is described in detail herein and its nucleotide sequence is set forth in Example 1 as SEQ ID NO:1. This nucleic acid molecule is referred to as "*ATPAC*". It is 36% identical and 51% similar to human *mdr1* across the entire sequence. It is 51% identical to the *atpgp1* gene reported by Dudler & Hertig (1997, *supra*) and 50% identical to *atpgp2*, a close homolog of *atpgp1*, published in the Genbank database. ATPAC protein is 65% similar to atpgp1 and atpgp2 proteins.

A partial clone of a *plPAC* of the invention was originally isolated from *Brassica napus* via differential expression screening of plants grown in the presence or absence of the chloride channel blocker, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB). A 0.5 kb gene fragment was identified, which had been up-regulated in response to NPPB treatment. This cDNA fragment was used to screen an *Arabidopsis* cDNA library, from which the complete *ATPAC* clone was isolated. The isolation and characterization of *ATPAC* is described in Example 1.

A genomic clone of *ATPAC* (SEQ ID NO:10) has also been isolated from a bacterial artificial chromosome (BAC) library of the *Arabidopsis* genome (BAC clone IGF F3J22, obtained from the *Arabidopsis* stock center, Ohio State University). A 7 kb fragment containing part of *ATPAC* and additional 5' regulatory sequences was subcloned into a plasmid vector (pBluescript). A restriction map of *ATPAC* is found in Fig. 3. The corresponding cDNA clone of *ATPAC* is found in SEQ ID NO:1 and its restriction map is Fig. 4.

In *Arabidopsis*, *plPAC* of the present invention is expressed in roots, leaves, flowers, and shoot meristem. Expression is strong in the hypocotyl of etiolated seedlings, but not in light grown seedlings. Expression of ATPAC is also relatively high in cotyledons, meristem, roots and the first true leaves of seedlings.

Among the unique features of this nucleic acid molecule as compared with other mdr-like genes from plants are its inducibility by certain compounds, including NPPB, herbicides and auxin, and its preferential expression in roots. The promoter regulatory region of *ATPAC* comprises

residues 1-3429 of SEQ ID NO:10.

Although the *ATPAC* cDNA clone from *Arabidopsis thaliana* is described and exemplified herein, this invention is intended to encompass nucleic acid sequences and proteins from other plant species that are sufficiently similar to be used instead of *ATPAC* nucleic acid and proteins for the purposes described below. These include, but are not limited to, allelic variants and natural mutants of SEQ ID NO:1, which are likely to be found in different species of plants or varieties of *Arabidopsis*.

Because such variants are expected to possess certain differences in nucleotide and amino acid sequence, this invention provides an isolated *plPAC* nucleic acid molecule having at least about 60% (preferably 70% and more preferably over 80%) sequence homology in the coding regions with the nucleotide sequence set forth as SEQ ID NO:1 or SEQ ID NO:10 (and, most preferably, specifically comprising the coding region of SEQ ID NO:1). Also provided are nucleic acids that encode a polypeptide that is at least about 40% (preferably 50% and most preferably 60%) similar to residues 1-76, 613-669 or 1144-1161 of SEQ ID NO:2. Also provided are nucleic acids that hybridize to the nucleic acids of SEQ ID NO:1, SEQ ID NO:10, or nucleic acids encoding the regions of residues 1-76, 613-669 or 1144-1161 of SEQ ID NO:2, preferably under moderate stringency (more preferably, high stringency, and most preferably, very high stringency).

In other preferred embodiments, the nucleic acids have a restriction digest map that is identical for at least 3 enzymes (more preferably 6 enzymes and most preferably 9 enzymes) to the maps shown in Figs. 3 or 4. In another

preferred embodiment, the nucleic acids have a restriction digest map identical to those shown in Fig. 3 for enzymes *Xho*I, *Xcm*I and *Spe*I (preferably additionally *Sac*I, *Pac*I and *Bsa*I, and most preferably additionally *Acl*I, *Ban*I and *Sna*BI).

In another preferred embodiment, the nucleic acids have a restriction digest map identical to those shown in Fig. 4 for enzymes *Xba*I, *Tat*I and *Nci*I (preferably additionally *Dra*I, *Bsm*I and *Bcl*I, and most preferably additionally *Acc*I, *Bsg*I and *Tli*I). The nucleic acids of the invention are at least 20 nucleic acids in length (preferably at least 50 nucleic acids and most preferably at least 100 nucleic acids).

In accordance with the invention, novel plPAC genes from two plant species, *Brassica napus* and *Arabidopsis thaliana*, are presented. This constitutes the first description of this unique p-glycoprotein in plants. Indeed, the closest known protein sequence, also from *Arabidopsis*, is only 65% identical suggesting that the *ATPAC* gene is novel and is expected to have novel properties. The isolation of two *plPAC* genes from different species enables the isolation of further *plPAC* genes from other plant species. Isolated nucleic acids that are *plPAC* genes from any plant species are considered part of the instant invention. In particular, the nucleic acids of other *plPAC* genes can be isolated using sequences of *ATPAC* that distinguish *plPAC* genes from other plant *mdr* genes according to methods that are well known to those in the art of gene isolation. In particular, sequences that encode residues 1-76, 613-669 and 1144-1161 of SEQ ID NO:2 can be used. In a preferred embodiment, the *plPAC* gene is from any higher plant species (more preferred from a dicot

species, and most preferred from a species in Brassicaceae (or Cruciferae)).

This invention also provides isolated polypeptide products of the open reading frames of SEQ ID NO:1 or SEQ ID NO:10, having at least about 70% (preferably 80% and most preferably 90%) sequence identity, or at least about 80% similarity (preferably 90% and more preferably 95%) with the amino acid sequence of SEQ ID NO:2. In another embodiment, the polypeptides of the invention are at least about 40% identical (preferably 50%, and most preferably 60%) to the regions of residues 1-76, 613-669 or 1144-1161 of SEQ ID NO:2. Because of the natural sequence variation likely to exist among *plPAC* genes, one skilled in the art would expect to find up to about 30-40% nucleotide sequence variation, while still maintaining the unique properties of the *plPAC* gene and encoded polypeptide of the present invention. Such an expectation is due in part to the degeneracy of the genetic code, as well as to the known evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the encoded protein. Accordingly, such variants are considered substantially the same as one another and are included within the scope of the present invention.

Also provided are transgenic plants transformed with part or all of the nucleic acids of the invention. Transgenic plants that over-express a plPAC coding sequence are one embodiment of this aspect of the invention. Example 3 provides for one prototype of such a plant. In a preferred embodiment, the *ATPAC* gene is used, and in a most preferred embodiment SEQ ID NO:1 or SEQ ID NO:10 is used. The *plPAC*

gene may be placed under a powerful constitutive promoter, such as the Cauliflower Mosaic Virus (CaMV) 35S promoter or the figwort mosaic virus 35S promoter. In a preferred embodiment, the 35SCaMV promoter is used. Transgenic plants expressing the *p1PAC* gene under an inducible promoter (either its own promoter or a heterologous promoter) are also contemplated to be within the scope of the present invention. Inducible plant promoters include the tetracycline repressor/operator controlled promoter. In a preferred embodiment, a native *p1PAC* promoter is used, and in a most preferred embodiment, residues 1-3429 of SEQ ID NO:10 is used. Plant species that are contemplated for overexpression of a *p1PAC* coding sequence include, but are not limited to, soybean.

In another embodiment, overexpression of *p1PAC* is induced to generate a co-suppression effect. This excess expression serves to promote down-regulation of both endogenous and exogenous *p1PAC* genes.

In some instances, it may be desirable to down-regulate or inhibit expression of endogenous *p1PAC* in plants possessing the gene. Accordingly, *p1PAC* nucleic acid molecules, or fragments thereof, may also be utilized to control the production of *p1PAC*-encoded P-glycoproteins. In one embodiment, full-length *p1PAC* antisense molecules or antisense oligonucleotides, targeted to specific regions of *p1PAC*-encoded RNA that are critical for translation, are used. The use of antisense molecules to decrease expression levels of a pre-determined gene is known in the art. In a preferred embodiment, antisense molecules are provided *in situ* by transforming plant cells with a DNA construct which,

upon transcription, produces the antisense sequences. Such constructs can be designed to produce full-length or partial antisense sequences. One example of antisense plPAC transgenic plants is given in Example 3.

In another embodiment, knock-out plants are obtained by screening a T-DNA mutagenized plant population for insertions in the *plPAC* gene (see Krysan et al., 1996, PNAS 93:8145). One example of this embodiment of the invention is found in Example 3. Optionally, transgenic plants can be created containing mutations in the region encoding the active site of *plPAC*. These last two embodiments are preferred over the use of anti-sense constructs due to the high homology among P-glycoproteins. The promoter of *ATPAC* is also provided in accordance with the invention. This promoter has the useful properties of root expression and inducability by NPPB. Presence of NPPB in the growth medium of *Arabidopsis* seedlings results in increased expression of *ATPAC* of the present invention.

Further, when approximately 4kb of upstream *ATPAC* promoter DNA is fused to the GUS reporter gene and transformed into wild-type plants, GUS staining is strong in the hypocotyl of etiolated seedlings, but not in light grown seedlings. Further, expression is high in cotyledons, meristem, root, and the first true leaves of seedlings. Staining was also observed in flowers and the apical portion of the inflorescence.

The prototypic example of this aspect of the invention is residues 1-3429 of SEQ ID NO:10. It is anticipated that plPAC genes from other plant species will likewise exhibit the aforementioned useful properties. As

these promoter regions can easily be isolated from the plPAC genes that are provided with the invention, all plant *plPAC* gene promoters are provided with the invention. The nucleic acids of the invention therefore include a nucleic acid molecule that is at least about 70% identical (preferably 80% and most preferably 90%) to the residues 1-3429 of SEQ ID NO:10. Also provided are nucleic acids that hybridize to the nucleic acid residues 1-3429 of SEQ ID NO:10 preferably under moderate stringency (more preferably, high stringency, and most preferably, very high stringency).

Thus, the *PlPAC* of the present invention encodes an ABC transporter that binds NPA and is involved with auxin transport in the plant. Mutants of *Arabidopsis* lacking *ATPAC* and double mutants lacking both *ATPAC* and *AtPGP1* display morphological phenotypes consistent with their demonstrated impairments in polar auxin transport. It has been widely accepted that NPA-sensitive regulatory site and the auxin-conducting channel of the efflux carrier are separate molecular entities. Strong evidence indicates that PIN-like genes encode the auxin-conducting channel of the efflux carrier (Palme and Galweiler (1999) Curr. Op. Plant Biol. 2:375-381). However, as evidenced in Example 4 of the present invention, MDR-like genes are components of the NPA-sensitive regulatory site.

Expression of the *plPAC* gene of the present invention is inducible by auxin as described below in Example 4. Treatment of wild type *Arabidopsis* seedlings with 2,4-D or high concentrations of auxin result in plants with the same phenotype as that of *ATPAC* mutant plants. The strongly curved organs of the double mutant resemble the twisted forms

induced by treatment of some dicots with herbicidal levels of the auxin analog, 2,4-D and in *Arabidopsis* by treatment with auxin transport inhibitors (Sieburth, (1999) Plant Physiol. 121: 1179-1190). This indicates that ATPAC of the present invention pumps auxin or auxin conjugates from sites of synthesis, such as the apical meristem and expanding cotyledons (Sachs, (1991) Development S1: 833-893). Under this model, tissues that express this pump would accumulate auxin as a result of the mutation and the altered auxin balance could be responsible for the altered growth patters typifying the *ATPAC* phenotype. Further support of this model is the similarity of auxin to indolylic substrates pumped by human MDR1, and the finding that *ATPAC* expression is increased by auxin. Also, the fact that NPA binds to ATPAC and that *atpac* knockout mutants can be phenocopied by auxin application suggests that ATPAC is an important component of the auxin transport and distribution machinery.

The present invention also provides antibodies capable of immunospecifically binding to polypeptides of the invention. In a preferred embodiment, the antibodies react immunospecifically with various epitopes of the *plPAC*-encoded polypeptides. In a particularly preferred embodiment, the antibodies are immunologically specific to the polypeptide of residues 1-76, 613-669 or 1144-1161 of SEQ ID NO:2.

The following sections sets forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et

al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (2001) (hereinafter "Ausubel et al.") are used.

III. Preparation of *PlPAC* Nucleic Acid Molecules, encoded Polypeptides, Antibodies Specific for the Polypeptides and Transgenic Plants

1. Nucleic Acid Molecules

PlPAC nucleic acid molecules of the invention may be prepared by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art.

The availability of nucleotide sequence information, such as the cDNA having SEQ ID NO:1, enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramadite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC). Long, double-stranded polynucleotides, such as a DNA molecule of the present invention, must be synthesized in stages, due to the size limitations inherent in current oligonucleotide synthetic methods. Thus, for example, a long double-stranded molecule may be synthesized as several smaller segments of appropriate complementarity. Complementary segments thus produced may be annealed such that each segment possesses

appropriate cohesive termini for attachment of an adjacent segment. Adjacent segments may be ligated by annealing cohesive termini in the presence of DNA ligase to construct an entire long double-stranded molecule. A synthetic DNA molecule so constructed may then be cloned and amplified in an appropriate vector.

PlPAC genes also may be isolated from appropriate biological sources using methods known in the art. In fact, the *ATPAC* clone was isolated from an *Arabidopsis* cDNA library using a partial clone obtained from *Brassica napus*. In alternative embodiments, genomic clones of *plPAC* may be isolated.

In accordance with the present invention, nucleic acids having the appropriate level sequence homology with part or all the coding regions of SEQ ID NO:1 or SEQ ID NO:10 may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al., using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 ¼g/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 2X SSC and 0.1% SDS; (4) 2 hours at 45-55°in 2X SSC and 0.1% SDS, changing the solution every 30 minutes. One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid

molecules of a specified sequence homology (Sambrook et al., 1989):

$T_m = 81.5°C + 16.6\text{Log}\,[Na+] + 0.41(\%\ G+C) - 0.63\ (\%\ \text{formamide}) - 600/\#\text{bp in duplex}$

As an illustration of the above formula, using [N+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

The stringency of the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are 20-25°C below the calculated T_m of the of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately 12-20°C below the T_m of the hybrid. In regards to the nucleic acids of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 ¼g/ml denatured salmon sperm DNA at 42°C, and wash in 2X SSC and 0.5% SDS at 55°C for 15 minutes. A high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 ¼g/ml denatured salmon sperm DNA at 42°C, and wash in 1X SSC and

0.5% SDS at 65°C for 15 minutes. A very high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 ¼g/ml denatured salmon sperm DNA at 42°C, and wash in 0.1X SSC and 0.5% SDS at 65°C for 15 minutes.

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid cloning/expression vector, such as pGEM-T (Promega Biotech, Madison, WI) or pBluescript (Stratagene, La Jolla, CA), either of which is propagated in a suitable *E. coli* host cell.

PlPAC nucleic acid molecules of the invention include cDNA, genomic DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of SEQ ID NO:1 or SEQ ID NO:10. Such oligonucleotides are useful as probes for detecting *plPAC* genes or mRNA in test samples, e.g. by PCR amplification, mapping of genes or for the positive or negative regulation of expression of *plPAC* genes at or before translation of the mRNA into proteins.

The *plPAC* promoter is also expected to be useful in connection with the present invention, inasmuch as it is inducible in plants upon exposure to anion channel blockers. As mentioned above, seven-kilobase fragment of genomic DNA has been isolated, which contains part or all of the *plPAC* promoter from *Arabidopsis thaliana*. This promoter can be

used in chimeric gene constructs to facilitate inducible expression of any coding sequence of interest, upon exposure to NPPB or similar-acting compounds.

2. Proteins and Antibodies

Polypeptides encoded by *plPAC* nucleic acids of the invention may be prepared in a variety of ways, according to known methods. If produced *in situ* the polypeptides may be purified from appropriate sources, e.g., plant roots or other plant parts.

Alternatively, the availability of nucleic acid molecules encoding the polypeptides enables production of the proteins using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate *in vitro* transcription vector, such a pSP64 or pSP65 for *in vitro* transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. *In vitro* transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland. According to a preferred embodiment, larger quantities of *plPAC*-encoded polypeptide may be produced by expression in a suitable procaryotic or eucaryotic system. For example, part or all of a DNA molecule, such as the cDNA having SEQ ID NO:1, may be inserted into a plasmid vector adapted for expression in a bacterial cell (such as *E. coli*) or a yeast cell (such as *Saccharomyces cerevisiae*), or into a baculovirus vector for expression in an insect cell. Such vectors comprise the regulatory elements necessary for expression of the DNA in the host cell, positioned in such a

manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

The plPAC polypeptide produced by gene expression in a recombinant procaryotic or eucyarotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used by skilled practitioners.

The *plPAC*-encoded polypeptides of the invention, prepared by the aforementioned methods, may be analyzed according to standard procedures.

Polyclonal or monoclonal antibodies directed toward any of the peptides encoded by *plPAC* may be prepared according to standard methods. Monoclonal antibodies may be prepared according to general methods of Köhler and Milstein, following standard protocols.

C. Transgenic Plants

Transgenic plants expressing the *plPAC* gene can be generated using standard plant transformation methods known to those skilled in the art. These include, but are not

limited to, *Agrobacterium* vectors, PEG treatment of protoplasts, biolistic DNA delivery, UV laser microbeam, gemini virus vectors, calcium phosphate treatment of protoplasts, electroporation of isolated protoplasts, agitation of cell suspensions with microbeads coated with the transforming DNA, direct DNA uptake, liposome-mediated DNA uptake, and the like. Such methods have been published in the art. See, e.g., Methods for Plant Molecular Biology (Weissbach & Weissbach, eds., 1988); Methods in Plant Molecular Biology (Schuler & Zielinski, eds., 1989); Plant Molecular Biology Manual (Gelvin, Schilperoort, Verma, eds., 1993); and Methods in Plant Molecular Biology - A Laboratory Manual (Maliga, Klessig, Cashmore, Gruissem & Varner, eds., 1994).

The method of transformation depends upon the plant to be transformed. The biolistic DNA delivery method is useful for nuclear transformation. In another embodiment of the invention, *Agrobacterium* vectors are used to advantage for efficient transformation of plant nuclei.

In a preferred embodiment, the gene is introduced into plant nuclei in *Agrobacterium* binary vectors. Such vectors include, but are not limited to, BIN19 (Bevan, 1984, Nucleic Acid Res 12: 8711-8721) and derivatives thereof, the pBI vector series (Jefferson et al., 1987, PNAS 83:844751), and binary vectors pGA482 and pGA492 (An, 1986) and others (for review, see An, 1995, Methods Mol Biol 44:47-58). In preferred embodiments, the pPZP211 vector (Hajdukiewicz et al., 1994, PMB 25:989994) or PCGN7366 (Calgene, CA) are used. DNA constructs for transforming a selected plant comprise a coding sequence of interest operably linked to appropriate 5'

(e.g., promoters and translational regulatory sequences) and 3' regulatory sequences (e.g., terminators).

DNA constructs for transforming a selected plant comprise a coding sequence of interest operably linked to appropriate 5' (e.g., promoters and translational regulatory sequences) and 3' regulatory sequences (e.g., terminators). In a preferred embodiment, the coding region is placed under a powerful constitutive promoter, such as the Cauliflower Mosaic Virus (CaMV) 35S promoter or the figwort mosaic virus 35S promoter. Other constitutive promoters contemplated for use in the present invention include, but are not limited to: T-DNA mannopine synthetase, nopaline synthase (NOS) and octopine synthase (OCS) promoters.

Transgenic plants expressing a sense or antisense SDS coding sequence under an inducible promoter are also contemplated to be within the scope of the present invention. Inducible plant promoters include the tetracycline repressor/operator controlled promoter, the heat shock gene promoters, stress (e.g., wounding)-induced promoters, defense responsive gene promoters (e.g. phenylalanine ammonia lyase genes), wound induced gene promoters (e.g. hydroxyproline rich cell wall protein genes), chemically-inducible gene promoters (e.g., nitrate reductase genes, glucanase genes, chitinase genes, etc.) and dark-inducible gene promoters (e.g., asparagine synthetase gene) to name a few.

Tissue specific and development-specific promoters are also contemplated for use in the present invention. Examples of these included, but are not limited to: the ribulose bisphosphate carboxylase (RuBisCo) small subunit gene promoters or chlorophyll a/b binding protein (CAB) gene

promoters for expression in photosynthetic tissue; the various seed storage protein gene promoters for expression in seeds; and the root-specific glutamine synthetase gene promoters where expression in roots is desired.

The coding region is also operably linked to an appropriate 3' regulatory sequence. In a preferred embodiment, the nopaline synthetase polyadenylation region (NOS) is used. Other useful 3' regulatory regions include, but are not limited to the octopine (OCS) polyadenylation region.

Using an *Agrobacterium* binary vector system for transformation, the *plPAC* coding region, under control of a constitutive or inducible promoter as described above, is linked to a nuclear drug resistance marker, such as kanamycin resistance. *Agrobacterium*-mediated transformation of plant nuclei is accomplished according to the following procedure:

(1) the gene is inserted into the selected *Agrobacterium* binary vector;

(2) transformation is accomplished by co-cultivation of plant tissue (e.g., leaf discs) with a suspension of recombinant *Agrobacterium*, followed by incubation (e.g., two days) on growth medium in the absence of the drug used as the selective medium (see, e.g., Horsch et al. 1985, Cold Spring Harb Symp Quant Biol. 50:4337);

(3) plant tissue is then transferred onto the selective medium to identify transformed tissue; and

(4) identified transformants are regenerated to intact plants.

It should be recognized that the amount of expression, as well as the tissue specificity of expression

of the *plPAC* gene in transformed plants can vary depending on the position of their insertion into the nuclear genome. Such position effects are well known in the art. For this reason, several nuclear transformants should be regenerated and tested for expression of the transgene.

IV. Uses of *PlPAC* Nucleic Acids, Encoded Proteins and Antibodies

1. *PlPAC* Nucleic Acids

PlPAC nucleic acids may be used for a variety of purposes in accordance with the present invention. The DNA, RNA, or fragments thereof may be used as probes to detect the presence of and/or expression of *plPAC* genes. Methods in which *plPAC* nucleic acids may be utilized as probes for such assays include, but are not limited to: (1) *in situ* hybridization; (2) Southern hybridization (3) northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR).

The *plPAC* nucleic acids of the invention may also be utilized as probes to identify related genes from other plant species. As is well known in the art and described above, hybridization stringencies may be adjusted to allow hybridization of nucleic acid probes with complementary sequences of varying degrees of homology. Thus, *plPAC* nucleic acids may be used to advantage to identify and characterize other genes of varying degrees of relation to the exemplary *ATPAC*, thereby enabling further characterization of this family of genes in plants. Additionally, they may be used to identify genes encoding

proteins that interact with the P-glycoprotein encoded by *p1PAC* (e.g., by the "interaction trap" technique).

Further, as described below, the genes or antisense molecules may be used to produce transgenic plants that have altered responses to herbicides and auxin.

2. P1PAC Proteins and Antibodies

Purified *p1PAC*-encoded P-glycoproteins, or fragments thereof, may be used to produce polyclonal or monoclonal antibodies which also may serve as sensitive detection reagents for the presence and accumulation of plant P-glycoproteins in cultured plant cells or tissues and in intact plants. Recombinant techniques enable expression of fusion proteins containing part or all of the *p1PAC*-encoded protein. The full length protein or fragments of the protein may be used to advantage to generate an array of monoclonal or polyclonal antibodies specific for various epitopes of the protein, thereby providing even greater sensitivity for detection of the protein in cells or tissue.

Polyclonal or monoclonal antibodies immunologically specific for *p1PAC*-encoded proteins may be used in a variety of assays designed to detect and quantitate the protein. Such assays include, but are not limited to: (1) flow cytometric analysis; (2) immunochemical localization in cultured cells or tissues; and (3) immunoblot analysis (e.g., dot blot, Western blot) of extracts from various cells and tissues.

Polyclonal or monoclonal antibodies that immunospecifically interact with one or more of the polypeptides encoded by *p1PAC* can be utilized for identifying

and purifying such proteins. For example, antibodies may be utilized for affinity separation of proteins with which they immunospecifically interact. Antibodies may also be used to immunoprecipitate proteins from a sample containing a mixture of proteins and other biological molecules.

3. plPAC Transgenic Plants

Transgenic plants that over- or under- express plPAC can be used in a varied of agronomic and research applications. From the foregoing discussion, it can be seen that *plPAC* and its homologs, and transgenic plants containing them will be useful for improving stress resistance or tolerance in plants. This provides an avenue for developing marginal or toxic soil environments for crop production. Both over- and under-expressing plPAC transgenic plants have great utility in the research of herbicides and other xenobiotic compounds.

As discussed above and in greater detail in Example 1, the similarity between plant and mammalian *mdr* genes indicates that their functional aspects will also be conserved. Thus, *plPAC* is expected to play an important role in the exclusion of toxic metabolic or xenobiotic compounds from cells. The fact that *plPAC* also is inducible and appears to be preferentially expressed in roots, where contact with such compounds often occurs, makes *plPAC* particularly desirable for genetic engineering of plants to increase their tolerance to such compounds. Accordingly, plants engineered to overexpress the *plPAC* gene should be resistant to a wide range of chemicals, both intentionally applied as herbicides or unintentionally as wastes. Examples

of the kinds of xenobiotics that should be detoxified by the *plPAC* of the invention include, but are not limited to, hydrophobic (i.e., lipophilic) herbicides and other compounds, such as 3(3,4-dichlorophenyl)-1,1, dimethyl urea (also known as DCMU or Diuron, available from Sigma Chemical Co., St. Louis, MO) or other hydrophobic compounds that disrupt photosynthetic electron transport, as well as Metachlor (Ciba Geigy, Basel Switzerland), Taurocholate (Sigma Chemical Co.), Primisulfuron (Ciba Geigy), and IRL-1803.

As illustrated in Example 2, plant cells that over-express a *plPAC* gene have surprisingly higher growth rate with or without the xenobiotic compound Rhodamine 6G. It is contemplated that *plPAC* overexpression may be a generally useful way to increase plant and plant cell culture growth, even without the presence of xenobiotic compounds.

In addition to the above-mentioned features and advantages of transgenic plants that are altered in their expression of *plPAC*, these plants will also be altered in auxin transport. Through the use of developmental or tissue specific promoters, plants having a pre-determined alteration in auxin transport may be produced, providing agronomically or horticulturally beneficial features to such plants.

The following specific examples are provided to illustrate embodiments of the invention. They are not intended to limit the scope of the invention in any way.

EXAMPLE 1
Cloning and Analysis of a *PlPAC* From *Arabidopsis thaliana*

The *plPAC* of the present invention was identified by its up-regulation in response to a chloride ion channel blocker. *Brassica napus* plants were grown either in the presence or absence of 20 ¼M 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB). After five days, the roots of the seedlings were harvested and total RNA was extracted separately from the treated and untreated plants. From the total RNA preparations, poly (A)+ RNA was isolated and used as the starting material to create a cDNA subtraction library, using the CLONTECH PCR-SELECT™ cDNA Subtraction Kit and accompanying instructions (CLONTECH Laboratories, Inc., Palo Alto, CA).

Using the subtractive hybridization kit, a gene fragment was identified that was up-regulated in response to treatment of the plants with NPPB. This fragment (0.5 kb) was used to screen a cDNA library of *Arabidopsis thaliana*, from which a full-length cDNA clone was isolated. The nucleotide sequence of this cDNA clone, referred to as *ATPAC* (*Arabidopsis thaliana* putative anion channel) is set forth below as SEQ ID NO:1.

The 3.76 kb cDNA clone encodes a polypeptide 1,254 amino acids in length. The deduced amino acid sequence encoded by SEQ ID NO:1 is shown in Figure 1 as "atpac" (SEQ ID NO:2), in a lineup with the following sequences: (1) hmdr1 (SEQ ID NO:3); (2) mmdr1 (SEQ ID NO:4); (3) hmdr3 (SEQ ID NO:5); (4) mmdr2 (SEQ ID NO:6); (5) atpgp1 (SEQ ID NO:7); and (6) atpgp2 (SEQ ID NO:8). A consensus sequence (SEQ ID NO:9) is also shown.

A search of various sequence databases indicates that *ATPAC* is a new and distinct member of the *mdr* family of

ABC transporters. In none of the databases, including the EST collection, does an exact match exist. The ABC transporter family is very large, consisting of at least two sub-groups, *mrp* and homologs and *mdr* and homologs. The only examples of plant *mdr*-like genes are *atpgp1* and *atpgp2* from *A. thaliana* and two homologs from potato and barley, respectively. Though the *atpgp1* and *atpgp2* genes are similar to *ATPAC*, they are only 51 and 50% identical, respectively, indicating that *ATPAC* is a distinct gene by comparison. Sequence homology with the potato and barley *mdr*-like genes is even more divergent. Another difference between the *agpgp1* gene and the *ATPAC* gene is their respective preferential expression in inflorescens and roots, respectively.

EXAMPLE 2
Effect of *ATPAC* Expression in Bacterial Cells on Their Ability to Detoxify Rhodamine 6G

The compound Rhodamine 6G is a well known substrate of mammalian p-glycoproteins (Kolaczkowski et al., J. Biol. Chem. 271: 31543-31548, 1996). The ability of a cell to detoxify the compound is indicative of activity of p-glycoproteins. A bacterial cell line was transformed with an expression vector comprising *ATPAC*. The growth rate of transformed and non-transformed cells was then measured, in the presence or absence of Rhodamine 6G. Results are shown in Figure 2. As can be seen, *ATPAC*-expressing cells grown in the absence of the drug had the best growth rate. Moreover, even in the presence of the drug, the cells grew more quickly than non-transformed cells in the presence or absence of

Rhodamine 6G. These results demonstrate that *ATPAC* encodes a functional and robust p-glycoprotein.

Example 3
Transgenic Plants that Overexpress and Underexpress ATPAC

Transformation construct. The *Agrobacterium* binary vector pPZP211 (Hajdukiewicz et al., 1994 Plant Mol. Biol. 25:989994) was digested with *EcoR*I and *Sma*I, and selfligated. This molecule was named pPZP211'. The *Agrobacterium* binary vector pCGN7366 (Calgene, CA) was digested with *Xho*I and cloned in SalIdigested pPZP211'. We named this binary vector pPZPPCGN. The 3.8 kb full-length *ATPAC* cDNA was cloned into the pGH19 vector. After digestion with *Sma*I (in the multiple cloning site upstream) and *EcoR*I, a 3.1 kb cDNA fragment was cut out. This *SmaIEcoR*I 3.1 kb fragment was cloned into the SmaI/EcoRI site of pPZPpCGN. The rest of *ATPAC* gene was amplified using polymerase chain reaction to have translationally fused HAtag at its 3'terminal. After ligating *EcoR*1 linkers to the ends of the resulting PCR product, the 0.7 kb fragment was cloned into the *EcoR*I site of the *SmaIEcoR*I 3.1 kb *ATPAC* fragment in pPZP-pCGN. The final construct was named pATPACOE.

Plant transformation. pATPACOE was introduced into *Agrobacterium tumefaciens* strain by a direct transformation method. *Agrobacterium*-mediated transformation was performed using vacuum infiltration (Bechtold et al., 1993,. CR Acad. Sci. [III] 316: 11941199.)

T1 plants which survived on kanamycincontaining plates were selected, transplanted into soil and grown to set

T2 seed. T3 seeds were collected from kanamycinresistant T2 plants. T3 plants which showed 100% kanamycinresistance were selected and were considered homozygous for the transgene.

Antisense Plants. The full length cDNA in pBluescript SK() vector (Stratagene, CA) is digested with *EcoR*I (there is a cleavage site in the upstream polylinker) and *Ssp*I. The resulting 1.3 Kb fragment representing a 5' portion of the *AtPAC* cDNA was cloned into the aforementioned pPZPPCGN, which had been digested with *EcoR*I/*Sma*I, ensuring that this fragment of the cDNA was inserted in the antisense orientation. This construct was named pATPACAE. pATPACAE was introduced into *Arabidopsis* plants by *Agrobacterium* transformation, as described above.

Knock-out Plants. The method of Krysan et al (1996, PNAS 93:8145, incorporated by reference herein) was followed using the following primers:

Genespecific primers:

AtpacF: CACTGCTCAATGATCTCGTTTTCTCACTA (SEQ ID NO:11)

AtpacR: CTTGAATCACACCAATGCAATCAACACCTC (SEQ ID NO:12)

Primers for TDNA left boarder:

JL202: CATTTTATAATAACGCTGCGGACATCTAC (SEQ ID NO:13)

JL270: TTTCTCCATATTGACCATCATACTCATTG (SEQ ID NO:14)

T-DNA insertion mutants were isolated by PCR-based screening of DNA pools. Two alleles of *ATPAC* and one *ATPGP1* allele were isolated. At the seedling stage, both alleles of *ATPAC* displayed down-folded, or epinastic cotyledons and abnormally angled cotyledon petioles. The petioles of the cotyledons and first true leaves in *ATPAC* were shorter than in wild type plants. As adult plants, the rosette leaves

were somewhat curled and wrinkled along the margin. Bolting of the inflorescence stem was delayed by 2.8 days on average, relative to wild-type. The bolt grew more slowly than wild type after starting, though the wild type length was utimately reached. These phenotypes coincided with the sites of expression indicated by GUS staining (as described herein). None of these phenotypes were present in plants transformed with a genomic fragment containing the wild type *ATPAC* promoter and coding sequence ("*atpac1-1*"). This mutant did not display any overt phenotype as a seedling or an adult plant. Double mutants were constructed by crossing *atpac1-1* and *atpgp1-1* plants. F1 individuals appeared wild type and were permitted to self pollinate. Approximately one in sixteen of the F2 seedlings displayed extremely down-curved cotyledons when grown in the light and also displayed shorter, wavy hypocotyls when grown in the dark. PCR analysis confirmed that these seedlings were homozygous double mutants. The adult double mutant plants were severely stunted in growth. Primary inflorescence stems of the double mutants and floral pedicels were also wavy in appearance, indicating that the direction of growth periodically changed during the elongation phase of these organs. After 72 hours of growth, the double mutant produced abundant secondary inflorescence stems, indicating a large reduction in apical dominance. Fertility of the flowers in the double mutants was poor, due to improper elongation of the stamen filament. Hand-pollinating flowers of the doubly homozygous mutant plants produced double-mutant seeds that were both viable and normal in appearance.

Example 4

Effect of Auxin (IAA) on *ATPAC* Expression

Expression in Yeast and Xenopus Oocytes. *ATPAC* cDNA was expressed in wild type yeast as well as in yeasts lacking seven ABC transporters (as described by Decottignies et al., (1998) J. Biol. Chem. 273:12612-22)in order to create a heterologous system for studying function of the transporter. The yeasts were exposed to toxic compounds that are known substrates for human MDR1. *ATPAC* did not confer any measurable resistance to the toxic substrates. Further, there was no evidence of a drug-pumping role for ATPAC.

In order to examine whether ATPAC functions as an anion chanel or a regulator of an anion channel, complementary RNA made from an *ATPAC* cDNA template was injected into Xenopus oocytes to produce a heterologous expression system amenable to electrophysiology. No currents associated with ATPAC were observed by two electrode voltage clamping.

Treatment of wild type seedlings with 2,4-D or high concentrations of auxin result in plants having strongly curved organs.

ATPAC Binds the Auxin Transport Inhibitor naphthylphthalamic acid (NPA). Yeast expressing ATPAC was assayed for NPA binding. NPA bound tightly and specifically to ATPAC-expressing yeast, but not to control yeast. Bound NPA was not displaced by benzoic acid, a related hydrophobic organic acid. Proteins that bind NPA and influence auxin transport are considered important regulators of hormone action and therefore, of plant development. The fact that

NPA binds to ATPAC and that *atpac* knockout mutants can be phenocopied by auxin application suggests that ATPAC is an important component of the auxin transport and distribution machinery.

Effect of ATPAC and AtPGP1 on Auxin Transport. Three different auxin transport assays of the Ws wild type, ATPAC-1, ATPAC-2 and atpgp1 were performed. The first measured the basipetal movement of auxin in seedlings as described by Murphy et al. (2000) Planta 211:315-324. A 0.1 µl microdroplet of radioactive auxin was placed on the apex of a light-grown seedling. Four hours later, the amount of radioactivity collected on moist filter paper that contacted the root tip was determined. Polar auxin transport measured in this manner was severely reduced in both alleles of *ATPAC* and especially in the double mutant, but not in the *atpgp1* mutant.

The second assay measured the basipetal transport of radioactive auxin through etiolated seedlings inverted in a reservoir containing radioactive auxin (Garbers et al. (1996) EMBO J. 15:2115-2124). The results were similar to those with the first assay described above.

The third assay measured basipetal transport of auxin in the inflorescence stem in a method described by Ruegger et al (1997) Plant Cell 9:745-57. A segment of inflorescence stem was excised and immersed apical-end down in a tube containing a small volume of radioactive auxin. At a later time, a piece of tissue was excised from the basal end of the segment and counted in a scintillation counter. Seedlings having *ATPAC* mutations clearly displayed defective auxin transport profiles. Further, the *atpgp1* mutation

significantly impaired transport in tissue segments taken from the lower portion of the inflorescence. This is indicative of gradients of function of at least two MDR-like gene products along the inflorescence axis.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.